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Docket No. CG372

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BOX PATENT APPLICATION
Assistant. Commissioner for Patents
Washington, DC 20231

Dear Sir:

Transmitted herewith for filing is the patent application of: Timothy Fowler and Stuart Causey
For: IMPROVED *ENTEROBACTERIACEAE* FERMENTATION STRAINS

Also enclosed are:

8 Sheets of Informal Drawings; Figs. 1-3
 Declaration and Power of Attorney - Executed; Unexecuted
 Sequence Listing
 Computer Readable Sequence Listing
 Statement of Sameness
 Information Disclosure Statement

The filing fee has been calculated as shown below:

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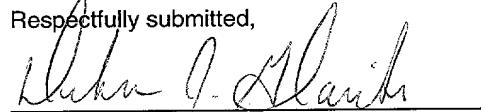
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Respectfully submitted,


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Date: June 23, 1997

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Date: June 23, 1997

By: Avis Ridenour
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Date: June 23, 1997

By: Avis Ridenour

Avis Ridenour

PATENT
Docket No. GC372

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IMPROVED *ENTEROBACTERIACEAE* FERMENTATION STRAINS

FIELD OF THE INVENTION

The present invention generally relates to improved fermentation strains of the family 10 *Enterobacteriaceae* and specifically to methods for modifying phenotypic characteristics of the strains relevant to growth conditions. In particular, the present invention relates to the identification of a cryptic plasmid found within a strain of *Enterobacteriaceae* which modulates mobilization properties of other resident plasmids while providing advantageous growth characteristics.

15

BACKGROUND OF THE INVENTION

There are numerous commercially important compounds in the carbohydrate pathway of bacterial strains of the family *Enterobacteriaceae* including among others 2-KLG, a precursor to ascorbic acid; idonic acid; L-gluconic acid; and 2,5-DKG. Biocatalytic processes 20 have been developed for the production of these compounds. In particular, Anderson et al., (1985, Science 230:144-149) disclose a metabolic pathway for *Erwinia herbicola* that permits the bioconversion of D-glucose to 2-KLG in a single fermentative step. In this bioconversion, there are a variety of intermediates and one step involves the reduction of 2,5-DKG to 2-KLG which is catalyzed by a recombinantly introduced NADPH-dependent 2,5-DKG reductase. In large scale fermentation of *Enterobacteriaceae* strains containing recombinantly introduced proteins, there remain concerns of a regulatory nature associated 25 with the recombinant nature of the organism.

Vladimir Dellic discloses a summary of a study of organisms for conversion of glucose to ketoacids on internet address "svibor@znanost.hr". This summary discloses the 30 presence in *Erwinia citreus* ATCC accession number 31623 of a plasmid of 3.8 kb, designated pPZG500. Dellic discloses the introduction of a tetracycline resistance gene into pPZG500 which was used to clone a 2,5-DKG reductase gene.

There remains a need to develop improved *Enterobacteriaceae* fermentation strains 35 that have desirable phenotypic characteristics relative to growth conditions and which minimize or eliminate the mobilization properties of resident plasmids under fermentation conditions.

SUMMARY OF THE INVENTION

The present invention generally relates to improved bacterial fermentation strains of the family *Enterobacteriaceae*. Specifically, the present invention relates to the identification and isolation of a 3.8 kb cryptic plasmid found in strains of 5 *Enterobacteriaceae*. In a preferred embodiment, the *Enterobacteriaceae* strain is a recombinant strain.

The present invention is based in part upon the unexpected discovery that elimination of the cryptic plasmid from the *Enterobacteriaceae* strain *Pantoea* allows for growth of the organism at higher temperatures, thereby decreasing the time for production of desired 10 compounds in the carbohydrate pathway. This discovery has the commercial benefit of potentially reducing both the capital cost and starting materials cost of large scale *Enterobacteriaceae* biocatalysis used in the production of desirable end-products, such as 2-keto-L-gluconic acid, a precursor of ascorbic acid.

The present invention is also based in part on the unexpected discovery that 15 elimination of the cryptic plasmid from *Pantoea* reduces the mobilization properties of *Pantoea* resident plasmids thereby creating a safer and more desirable fermentation strain for the production of materials ultimately intended for human consumption, such as ascorbic acid.

The present invention provides a method for preparing an improved 20 *Enterobacteriaceae* strain from a progenitor *Enterobacteriaceae* strain containing a cryptic plasmid, comprising the step of eliminating the cryptic plasmid from the progenitor strain thereby creating the improved strain. Preferably, the nucleic acid sequence of a 3.8 kb cryptic plasmid according to the invention comprises the plasmid designated herein as pS. The nucleic acid sequence of pS is provided herein and provides a means for identifying 25 plasmids according to the invention which exist in *Enterobacteriaceae* species. The present invention also provides a method for reducing the mobilization properties of plasmids residing within a progenitor *Enterobacteriaceae* strain containing a cryptic plasmid comprising the step of eliminating part or all of the cryptic plasmid from the strain. In an alternative embodiment, the cryptic plasmid nucleic acid is mutated via recombinant DNA 30 techniques to reduce the mobilization properties and/or produce the desirable growth characteristics.

In another embodiment, the present invention provides for an isolated nucleic acid having the sequence as shown in SEQ ID NO:1 and NO:2. In another embodiment, the present invention provides for an isolated amino acid as shown in SEQ ID NO:3. In yet 35 another embodiment, the present invention provides a recombinant host cell produced by methods of the present invention. In one embodiment of the present invention, the host cell

is an *Enterobacteriaceae* strain and in another embodiment, the host cell is *Pantoea citrea* having ATCC accession number 31940.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figures 1A-1F illustrates the nucleic acid sequence of cryptic plasmid designated herein as pS. The nucleic acid sequence is illustrated in two halves, SEQ ID NO: 1, Figures 1A-1C and SEQ ID NO: 2 Figures 1D-1F. The amino acid sequence corresponding to the largest open reading frame (SEQ ID NO:3) is translated in Figures 1A-1C.

10 Figure 2 illustrates the growth at 28°C, 32°C, and 36°C in ML5 media of a strain containing the cryptic plasmid (pS+) vs a strain that has been cured of the cryptic plasmid (pS-).

15 Figure 3 illustrates the growth at 28°C, 32°C, and 36°C in Luria broth of a strain containing the cryptic plasmid (pS+) vs a strain that has been cured of the cryptic plasmid (pS-).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

20 As used herein, the family "*Enterobacteriaceae*" refers to fermentative bacterial strains having the general characteristics of being gram negative, oxidase-negative and being facultatively anaerobic. Preferred *Enterobacteriaceae* strains are those that are able to produce 2,5-diketo-D-gluconic acid from D-glucose solutions. Included in the family of *Enterobacteriaceae* which are able to produce 2,5-diketo-D-gluconic acid from D-glucose 25 solutions are the genus *Erwinia*, *Enterobacter*, *Gluconobacter* and *Pantoea*, for example. Compounds of interest in the microbial carbohydrate pathway, include but are not limited to D-gluconate (GA), 2-keto-D-gluconate (2KDG), 2,5-diketo-D-gluconate (2,5DKG), 5DKG, 2-keto-L-gluconic acid (2KLG), L-idonic acid (IA) and ascorbic acid. In the present invention, a preferred *Enterobacteriaceae* fermentation strain is *Pantoea citrea* and preferred 30 end compounds include idonic acid, 2KLG and ascorbic acid.

It is well understood in the art that the acidic derivatives of saccharides, may exist in a variety of ionization states depending upon their surrounding media, if in solution, or out of solution from which they are prepared if in solid form. The use of a term, such as, for example, gluconic acid, to designate such molecules is intended to include all ionization 35 states of the organic molecule referred to. Thus, for example, both "D-gluconic acid" and "D-gluconate" refer to the same organic moiety, and are not intended to specify particular

ionization states. The present invention encompasses the unionized forms of derivatives of saccharides, such as, for example, the sodium, potassium or other salt.

As used herein, the phrase "progenitor strain" refers to an *Enterobacteriaceae* strain containing a cryptic plasmid. The term "cryptic plasmid" refers to a plasmid found naturally occurring in a *Enterobacteriaceae* strain which when deleted from the progenitor strain alters the phenotypic growth characteristics or alters mobilization properties of other *Enterobacteriaceae* resident plasmids.

A preferred cryptic plasmid is designated herein as "pS" and refers to a 3.8 kb nucleic acid having the sequence as depicted in SEQ ID NO:1 and SEQ ID NO:2. However, the present invention further encompasses homologs and variations of SEQ ID NO:1 and SEQ ID NO:2 that retain at least one functional characteristic associated with SEQ ID NO:1 and NO:2, i.e., improved phenotypic growth characteristics or reduction of resident plasmid mobilization properties. Due to the degeneracy of the genetic code, a variety of nucleic acids could encode a deduced amino acid sequence encoded by an open reading frame present in SEQ ID NO: 1 and SEQ ID NO:2. Cryptic plasmids according to the present invention encompass all such nucleic acid variations.

Preferred nucleic acid homologs or variations are those having at least 80%, at least 90% and at least 95% identity to SEQ ID NO: 1 and SEQ ID NO:2. Preferred nucleic acid homologs or variations hybridize under high stringency conditions. The deduced amino acid sequence (SEQ ID NO:3) encoded by the open reading frame shown in SEQ ID NO:1 is illustrated in Figures 1A-1C.

As used herein, the term "recombinant" refers to an *Enterobacteriaceae* strain that contains nucleic acid not naturally occurring in the strain which has been introduced into the strain using recombinant techniques.

As used herein, the term "improved" when referring to an industrial fermentation strain means a strain having at least one desirable phenotypic modification of the progenitor strain. Illustrative of such desirable phenotype modifications include ability to grow at higher temperatures, increased growth rate upon fermentation at higher temperatures and loss of mobilization of plasmids residing within the strain.

The property of "mobilization" as used herein refers to the transmissibility of a plasmid residing within a fermentation strain.

As used herein the phrase "eliminating the cryptic plasmid from the fermentation strain" refers to the process of curing a fermentation strain of a cryptic plasmid. The present invention also encompasses modifications of the cryptic plasmid nucleic acid made through recombinant means, such as deletions, insertions, mutations, which interrupt the plasmid and its function in the host cell.

Oxidative enzymes associated with the biocatalysis of D-glucose to pathway intermediates include D-glucose dehydrogenase, D-gluconate dehydrogenase and 2-keto-D-gluconate dehydrogenase. Reductive enzymes associated with the biocatalysis of pathway intermediates into desired end-products include 2,5-diketo-D-gluconate reductase (DKGR), 5 2-keto reductase (2-KR) and 5-keto reductase (5-KR). Such enzymes include those produced naturally by the host strain or those introduced via recombinant means.

As used herein, the term "heterologous" refers to proteins such as enzymes that are not naturally present in host industrial fermentation strains but which have been introduced through recombinant DNA technology.

10 As used herein, the term fermentation refers to the range of 10 L to 500,000 L cultures.

Description of the Preferred Embodiments

The present invention relates to the identification and isolation of a cryptic plasmid 15 from a strain of the family *Enterobacteriaceae*. Eliminating this plasmid from the *Enterobacteriaceae* strain reduced the mobilization properties of a resident plasmid, allowed the organism to grow at higher temperatures than the progenitor strain and provides a means for producing desirable end products in shorter fermentation runs thereby reducing the cost of their production. The following is a description of a preferred embodiment. The techniques 20 disclosed herein are applicable to other embodiments of the present invention.

I. Biocatalysis

The present invention encompasses strains of the family *Enterobacteriaceae*. Preferred strains of the family *Enterobacteriaceae* are those that produce 2,5-diketo-D- 25 gluconic acid from D-glucose solutions, including *Pantoea*, are described in Kageyama et al. (1992, International Journal of Systematic Bacteriology vol. 42, p. 203-210). The metabolic pathway of carbohydrate metabolism in recombinant 2-KLG producing strains is disclosed in Lazarus et al. (1990, Proceedings 6th international Symposium on Genetics of Industrial Microorganisms, Strasbourg, Vol. II 1073-1082).

30 Biocatalysis begins with a suitable carbon source ordinarily used by *Enterobacteriaceae* strains, such as glucose. Other metabolite sources include, but are not limited to galactose, lactose, fructose, or the enzymatic derivatives of such. In addition to an appropriate carbon source, fermentation media must contain suitable minerals, salts, cofactors, buffers and other components, known to those of skill in the art for the growth of 35 cultures and promotion of the enzymatic pathway necessary for production of desired end-products.

In one illustrative *Pantoea* pathway, D-glucose undergoes a series of oxidative steps through enzymatic conversions, which may include the enzymes D-glucose dehydrogenase, D-gluconate dehydrogenase and 2-keto-D-gluconate dehydrogenase to give intermediates which may include, but are not limited to GA, 2KDG, and 2,5-DKG, see United States Patent 5 3,790,444. These intermediates undergo a series of reducing steps through enzymatic conversions, which may include the enzymes 2,5-diketo-D-gluconate reductase (DKGR), 2-keto reductase (2-KR) and 5-keto reductase (5-KR) to give end products which include but are not limited to 2KLG and IA.

The present invention also encompasses other metabolic pathways and intermediates 10 naturally occurring in or recombinantly introduced into *Enterobacteriaceae* strains, such as for example, a pathway that proceeds through the intermediate sorbitol.

In a preferred embodiment of the present invention, the preferred fermentation strain is *Pantoea citrea*, ATCC accession number 39140. The present invention encompasses the 15 production of desired pathway end products obtained entirely through *in vivo* methods or combined *in vivo/in vitro* methods.

II. Recombinant Introduction of Enzymes into Fermentation Strains

Any enzymes necessary for directing a *Enterobacteriaceae* strain carbohydrate pathway into desired end-products can be introduced via recombinant DNA techniques 20 known to those of skill in the art if such enzymes are not naturally occurring. Alternatively, enzymes that would hinder a desired pathway can be deleted by recombinant DNA methods. The present invention includes the recombinant introduction or deletion of any enzyme or intermediate necessary to achieve a desired pathway. As used herein, recombinant DNA technology includes *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* 25 recombinant/genetic recombination. See, for example, the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing “Associates and Wiley Interscience, N.Y.

In one embodiment of the present invention, nucleic acid encoding DKGR is 30 recombinantly introduced into the *Pantoea* fermentation strain. Many species have been found to contain DKGR particularly members of the *Coryneform* group, including the genera *Corynebacterium*, *Brevibacterium*, and *Arthrobacter*.

In one preferred embodiment of the present invention, 2,5-DKGR from 35 *Corynebacterium* sp. strain SHS752001 (Grindley et al., 1988, Applied and Environmental Microbiology 54: 1770-1775) is recombinantly introduced into a *Pantoea citrea* and the desired end product is 2KLG, a precursor to ascorbic acid. Production of recombinant 2,5

DKG reductase by *Erwinia herbicola* is disclosed in United States Patent No. 5,008,193 to Anderson et al.

A preferred plasmid for the recombinant introduction of non-naturally occurring enzymes or intermediates into a strain of *Enterobacteriaceae* is RSF1010, a mobilizable, but not self transmissible plasmid which has the capability to replicate in a broad range of bacterial hosts, including Gram - and Gram+ bacteria. (Frey et al., 1989, *The Molecular biology of IncQ plasmids*. In: Thomas (Ed.), Promiscuous Plasmids of Gram Negative Bacteria. Academic Press, London, pp. 79-94). Frey et al. (1992, *Gene* 113:101-106) report on three regions found to affect the mobilization properties of RSF1010. In a preferred embodiment, mobilization defective RSF1010 mutants are used for the recombinant introduction of non-naturally occurring enzymes into *Enterobacteriaceae* strains that have been cured of a cryptic plasmid.

III. Growth Conditions

Typically *Enterobacteriaceae* host cells are grown in the range of about 28°C to about 37°C in appropriate culture media. General growth conditions are disclosed in Truesdell et al., (1991, *Journal of Bacteriology*, 173: 6651-6656) and Sonoyama et al. (1982, *Applied and Environmental Microbiology*, vol. 43, p. 1064-1069). *Pantoea citrea* ATCC accession number 39140 has a nicotinic acid growth requirement which can be provided by nicotinomide at 100 µg/ml (Sigma). Culture media may be supplemented by the presence of selectable markers such as antibiotic resistance genes, including but not limited to tetracycline, ampicillin or chloramphenicol.

Three physical parameters which affect fermentation performance include dissolved oxygen content, pH and temperature. The rate of oxidation of metabolite sources can be limited by oxygen availability. However, the oxidation reaction will go to completion as long as oxygen is continually supplied to the culture media. In *Pantoea citrea* fermentation, air is continually provided to the fermentation tanks.

The genera *Pantoea* maintains metabolic activity under a wide range of pH conditions. Preferred pH is in the range of 4.0 to 7.5. Table 1 discloses the pH optimum for desired pathway conditions of *Pantoea citrea*.

TABLE 1	
Condition	pH Opt.
1. Growth	6.0 - <7.5
Glucose to 2-KDG (Ox.)	4.0-5.5
2-KDG to 2,5 DKG (Ox.)	5.0-5.5
2,5-DKG to 2-KGA (Red.)	5.5-6.0
2-KGA to Idonate (Red.)	6.5-7.5
Idonate to 2-KGA (Ox.)	5.5-6.5

Several combinations of acid and base can be used for pH control, including but not limited to, phosphoric acid, sulfuric acid, hydrochloric acid, sodium bicarbonate, calcium 5 carbonate, sodium hydroxide, ammonium hydroxide, and calcium hydroxide.

The optimum growth temperature of *Pantoea* citrea ATCC accession number 39140 containing the cryptic plasmid is 28 to 30°C. In ATCC accession number 39140 that has been cured of the cryptic plasmid, growth can occur at temperatures above 30°C up to about 36°C. Figures 2 and 3 illustrate the growth characteristics at 28°C, 32°C, 36°C of a pS+ 10 strain vs a pS- strain. The growth differential between pS+ and pS- strains appears more pronounced in minimal media conditions. Accordingly, the present invention provides the unexpected advantage of providing a method for producing improved fermentation strains that can grow at higher temperatures under minimal media conditions, thereby reducing the overall cost of fermentation, i.e., production of end products. Because the kinetic parameters 15 for various enzymes in the carbohydrate metabolism pathway may be affected by elevated temperatures, the relative ratios of end-products may be affected. Accordingly, another unexpected advantage of culturing a pS- strain under elevated temperatures is to affect a shift in the ratios of end-products.

Biocatalysis end products can be measured by HPLC analysis of the fermentation 20 broth with standard concentrations being used as controls.

IV. Nucleic Acid Identification Methods

Means for identifying a cryptic plasmid nucleic acid within a *Enterobacteriaceae* species include hybridization screening techniques that use radioactive or enzymatically 25 labeled probes to screen the suspect species nucleic acid under high stringency conditions. The term probe refers to a portion, fragment or segment of SEQ ID NO:1 or SEQ ID NO:2 that is capable of being hybridized to a desired target nucleotide sequence and probes can be used to detect, amplify or quantify nucleic acid. Probes may be labeled by nick translation, Klenow fill-in reaction, PCR or other methods well known in the art.

Alternatively, polymerase chain reaction (PCR) based strategy (United States Patents 4,683,195; 4,800,195; 4,965,188) may be used to identify nucleic acid of the cryptic plasmid. Oligonucleotides preferably in the range of 18-22 nucleotides derived from SEQ ID NO:1 or SEQ ID NO:2 serve as primers for the PCR reaction with the template comprising 5 nucleic acid derived from suspect *Pantoea* species. Any PCR products or other identified nucleic acid may be subcloned and subjected to nucleic acid sequencing to confirm the identity of the cryptic plasmid.

Methods for nucleic acid sequencing are well known in the art. Conventional enzymatic methods employ DNA polymerase Klenow fragment, SEQUENASE® (US 10 Biochemical Corp, Cleveland, OH) or Taq polymerase to extend DNA chains from an oligonucleotide primer annealed to the DNA template of interest.

The cryptic plasmid pS was sequenced in two parts and is shown in SEQ ID NO:1 and SEQ ID NO:2. Scientific programs such as, DNASTAR (DNASTAR Inc., 1228 South Park St., Madison, WI 53715) are available to those of skill in the art for joining the two 15 sequences, SEQ ID NO:1 and SEQ ID NO:2, in order to obtain a continuous sequence.

V. Methods for Curing *Enterobacteriaceae* Strains of the Cryptic Plasmid

Methods for curing the cryptic plasmid from *Enterobacteriaceae* are described in Jeffrey Miller (1972, in Curing of Episomes from E.Coli strains with Acridine Orange from 20 Experiments in Molecular Genetics, Cold Spring Harbor Laboratories, pg. 140). In this method, acridine orange is added to 5 ml cultures of an *Enterobacteriaceae* strain at 125 µg/ml and allowed to grow overnight at 37 °C. The following day, the cultures are plated out and individual colonies are used to prepare plasmid nucleic acid. The nucleic acid is analysed by means known to those of skill in the art to determine the presence or absence of 25 the plasmid.

The manner and method of carrying out the present invention may be more fully understood by those of skill in the art by reference to the following examples, which examples are not intended in any manner to limit the scope of the present invention or of the claims directed thereto.

30

EXAMPLES

I. Identification of the Cryptic Plasmid.

This example describes the initial discovery and identification of pS in *Pantoea* citrea. pS was discovered during a plasmid purification experiment specifically designed to 35 find any cryptic plasmids native to the *Pantoea* citrea.

Pantoea citrea ATCC accession number 39140 was subjected to a plasmid preparation by standard means. Plasmid DNA was subjected to 1% agarose gel electrophoresis and a plasmid of 3.8kb, designated pS, was identified. The 3.8 kb band was excised and the nucleic acid was electroeluted and purified by precipitation. pS nucleic acid 5 was subjected to restriction analysis via restriction endonucleases and sequenced by standard dideoxy sequencing methodology. The nucleic acid sequence of pS is shown in two halves, SEQ ID NO:1 and SEQ ID NO:2. SEQ ID NO:1 and SEQ ID NO:2 were subjected to a BLAST search (FastA Genetics Computer Group, Inc., University Research Park, 575 Science Dr. Ste. B, Madison, WI 53711) which revealed no homology to known nucleic 10 acids. The amino acid sequence encoding by the largest open reading frame is shown in Figures 1A - 1C and is designated SEQ ID NO:3.

II. Curing Pantoea citrea of pS.

This example describes the method for curing *Pantoea citrea* of pS. 15 Acridine orange was added to 5 ml cultures of *Pantoea citrea* ATCC accession number 39140 at 125 µg/ml and allowed to grow overnight at 37 °C. The following day, the cultures were plated out and individual colonies were used to prepare plasmid nucleic acid. The nucleic acid thus prepared was analysed on a 1% agarose gel to determine the presence or absence of pS. One colony was determined to no longer contain pS. Subsequent 20 purification of the colony and repeated efforts to isolate the plasmid DNA confirmed that this particular isolate had lost the cryptic plasmid. This culture, designated as *Pantoea citrea* pS- or "pS-" was used for subsequent experimentation in comparisons with *Pantoea citrea* containing pS or "pS+"

25 III. Transmissibility of Vector Plasmids from Pantoea citrea to other Microbial Hosts.

The purpose of this example was to determine the transfer frequency of expression vectors from *Pantoea citrea* to *Escherichia.coli* and *Pseudomonas aeruginosa*. Transfer frequency was assayed by mating strains of *Pantoea citrea* with *E.coli* and *P.aeruginosa* under selective conditions and in the presence of a counter selective agent or condition. 30 An expression vector containing nucleic acid encoding a DGKR was created using plasmid RSF1010 which was modified by deleting a region of the plasmid involved in mobilization (Frey et al., *supra*). Additionally, the cryptic plasmid, was cured from *Pantoea citrea* by the method disclosed in Example II.

Materials and Methods

E.coli strain 294 (endA, hsdR, Thi⁻, Pro⁻, Str^S) was used as the recipient in all the experiments pertaining to E.coli. Selective conditions included Tetracycline at 20 and 100 $\mu\text{g}/\text{ml}$ and Streptomycin at 100 and 500 $\mu\text{g}/\text{ml}$. Counter selective agents or conditions 5 include growth at 42 °C. or the presence of Irgasan at 12 $\mu\text{g}/\text{ml}$. The selective temperature conditions were chosen to be 37°C, however, it was determined that the two *Pantoea citrea* strains having been cured of the cryptic plasmid were able to grow at 37°C. the *Pantoea citrea* strain that contained the cryptic plasmid (pS-) was still unable to grow at 37°C. Accordingly, all *P. citrea* strains were tested for growth at 42°C. All three *P. citrea* strains 10 failed to grow at this temperature under the conditions used for selection, while the recipient *E. coli* strain was able to grow at this temperature. The same conditions were used for *P. aeruginosa* as used for *E. coli*. Table II illustrates the selective and counterselective conditions used for experimentation.

TABLE 2. SELECTIVE AND COUNTERSELECTIVE AGENTS AND CONDITIONS

MATING	SELECTIVE ANTIBIOTIC	COUNTER SELECTIVE AGENT OR CONDITION
<i>P. citrea</i> -pS (pD92) X <i>E. coli</i> 294	Tetracycline 20 $\mu\text{g}/\text{ml}$	Growth @ 42°C
<i>P. citrea</i> -139-2A (Δ 18) X <i>E. coli</i> 294	Streptomycin 100 $\mu\text{g}/\text{ml}$	Growth @ 42°C
<i>P. citrea</i> -pS (Δ 18) X <i>E. coli</i> 294	Streptomycin 100 $\mu\text{g}/\text{ml}$	Growth @ 42°C
<i>P. citrea</i> -pS (pD92) X <i>P. aeruginosa</i> PA01	Tetracycline 100 $\mu\text{g}/\text{ml}$	Irgasan 12 $\mu\text{g}/\text{ml}$
<i>P. citrea</i> -139-2A (Δ 18) X <i>P. aeruginosa</i> PA01	Streptomycin 500 $\mu\text{g}/\text{ml}$	Irgasan 12 $\mu\text{g}/\text{ml}$
<i>P. citrea</i> -pS (Δ 18) X <i>P. aeruginosa</i> PA01	Streptomycin 500 $\mu\text{g}/\text{ml}$	Irgasan 12 $\mu\text{g}/\text{ml}$

15

Mating

Each strain of *Pantoea citrea* was mated with *E.coli* 294 and *P. aeruginosa* PA01 on three separate occasions. As a negative control, each strain of *P. citrea* was used in a mock mating without a recipient. No growth was observed on any of the selective plates in these 20 mock matings.

The frequency of transfer was calculated as follows:

$$\text{Frequency of transfer (\%)} = \frac{\# \text{ Transconjugants/ml}}{\# \text{ Input Donor/ml}} \times 100$$

The data from all matings are shown in Table 3.

TABLE 3. FREQUENCY OF TRANSMISSION OF VECTOR PLASMIDS TO <i>E. COLI</i> 294 AND <i>P. AERUGINOSA</i> PA01				
Mating	Exp. 1	Exp. 2	Exp. 3	Mean
<i>P. citrea</i> -pS (pD92) X <i>E. coli</i> 294	.001%	.0003%	.0008%	.0007%
<i>P. citrea</i> -139-2A (Δ18) X <i>E. coli</i> 294	.0017%	.0002%	.0005%	.0008%
<i>P. citrea</i> -pS (Δ18) X <i>E. coli</i> 294	.0009%	.0002%	.00007%	.0004%
<i>P. citrea</i> -pS (pD92) X <i>P. aeruginosa</i> PA01	.0006%	.0001%	.00003%	.0002%
<i>P. citrea</i> -139-2A (Δ18) X <i>P. aeruginosa</i> PA01	.0001%	.0003%	.0001%	.0002%
<i>P. citrea</i> -pS (Δ18) X <i>P. aeruginosa</i> PA01	.0003%	.00015%	.0001%	.0002%

Analysis of Transconjugants

5 Because of the very low apparent frequency of transmission that was observed genetically, it was necessary to examine transconjugants for the presence of vector plasmids to determine whether transmission had actually occurred or whether the colonies observed on the selection plates were due to selection of chromosomal mutations that result in resistance to the antibiotics used. 10 transconjugants from each mating were examined for the presence 10 of plasmids. Plasmids were detected in all cases.

Results

The results shown in Table 3 indicate that transmission of the expression vector from *P. citrea* cured of the small cryptic plasmid (pS-) to *E. coli* and *P. aeruginosa* has been reduced 15 about 1000 fold in comparison to *P. citrea* having the small cryptic plasmid present.

Transmission of RSF1010 based expression vector having the deletion disclosed in Frey et al., supra, to *E. coli* and *P. aeruginosa* from *P. citrea* was reduced by about 1000 fold in comparison to transmission of the RSF1010 plasmid without the mutation to these organisms from *P. citrea*.

20 Various other examples and modifications of the foregoing description and examples will be apparent to a person skilled in the art after reading the disclosure without departing from the spirit and scope of the invention, and it is intended that all such examples or modifications be included within the scope of the appended claims. All publications and patents referenced 25 herein are hereby incorporated in their entirety.

WHAT IS CLAIMED IS:

1. A method for preparing an improved *Enterobacteriaceae* strain from a progenitor *Enterobacteriaceae* strain containing a cryptic plasmid comprising the step of eliminating the 5 cryptic plasmid from the progenitor strain thereby creating the improved strain.
2. The method of Claim 1 wherein the progenitor strain is selected from the group consisting of *Pantoea*, *Enterobacter*, *Erwinia* and *Gluconobacter*.
- 10 3. The method of Claim 2 wherein the *Pantoea* strain is *Pantoea citrea*.
4. The method of Claim 1 wherein the progenitor strain is capable of producing 2,5-DKG from a carbon source.
- 15 5. The method of Claim 1 wherein the progenitor strain is a recombinant strain that comprises nucleic acid encoding heterologous 2,5-DKGR and is capable of converting 2,5-DKG to 2-KLG.
- 20 6. The method of Claim 1 wherein the cryptic plasmid is pS having the nucleic acid sequence as shown in SEQ ID NO:1 or SEQ ID NO:2.
7. The method of Claim 3 wherein *Pantoea citrea* has ATCC accession number 31940.
- 25 8. The method of Claim 1 wherein the improved stain is able to grow at higher temperatures than the progenitor strain.
9. A method for reducing the mobilization properties of plasmids residing within an *Enterobacteriaceae* strain containing a cryptic plasmid, comprising the step of eliminating 30 the cryptic plasmid from the strain.
10. The method of Claim 9 wherein the *Enterobacteriaceae* strain produces 2,5-DKG.

11. The method of Claim 10 wherein the *Enterobacteriaceae* strain is a recombinant strain that comprises nucleic acid encoding heterologous 2,5-DKGR and is able to convert 2,5-DKG to 2-KLG.

5 12. The method of Claim 9 wherein the *Enterobacteriaceae* strain is selected from the group consisting of *Pantoea*, *Enterobacter*, *Erwinia* and *Gluconobacter*.

13. The method of Claim 12 wherein the *Pantoea* strain is *Pantoea citrea*.

10 14. The method of Claim 9 wherein the cryptic plasmid is pS having the nucleic acid sequence as shown in SEQ ID NO:1 or SEQ ID NO:2.

15. The method of Claim 13 wherein *Pantoea citrea* has the ATCC accession number 31940.

15 16. An improved *Enterobacteriaceae* strain made by the method of Claim 1.

17. The improved *Enterobacteriaceae* strain of Claim 16 that is *Pantoea*.

20 18. An isolated nucleic acid having the sequence as shown in SEQ ID NO:1.

19. An isolated nucleic acid having the sequence as shown in SEQ ID NO:2.

20. An isolated amino acid having the sequence as shown in SEQ ID NO:3.

25

ABSTRACT

Methods are provided for preparing improved fermentation strains of the family *Enterobacteriaceae* which comprise the steps of eliminating the cryptic plasmid from the progenitor strain thereby creating the improved strain. Methods for reducing the 5 mobilization properties of resident plasmids in an *Enterobacteriaceae* strain containing a cryptic plasmid are also provided. The present invention provides the nucleic acid sequence of pS, a cryptic plasmid found in *Pantoea* which can be used to identify the cryptic plasmid in strains of *Enterobacteriaceae*.

10

He was a man of great energy and determination, and he was always looking for ways to improve the quality of life for the people of his community. He was a true leader, and his legacy will continue to inspire others for years to come.

DECLARATION
AND POWER OF ATTORNEY

ORIGINAL APPLICATION

DOCKET INFORMATION

GC372

AS A BELOW NAMED INVENTOR, I HEREBY DECLARE THAT:

MY RESIDENCE, POST OFFICE ADDRESS AND CITIZENSHIP ARE AS STATED BELOW NEXT TO MY NAME. I BELIEVE I AM THE ORIGINAL, FIRST AND SOLE INVENTOR (IF ONLY ONE NAME IS LISTED BELOW) OR AN ORIGINAL, FIRST AND JOINT INVENTOR (IF PLURAL NAMES ARE LISTED BELOW) OF THE SUBJECT MATTER WHICH IS CLAIMED AND FOR WHICH A PATENT IS SOUGHT ON THE INVENTION ENTITLED "IMPROVED ENTEROBACTERIACEAE FERMENTATION STRAINS", THE SPECIFICATION OF WHICH

CHECK ONE:

IS ATTACHED HERETO
 WAS FILED ON JUNE 23, 1997

AS APPLICATION SERIAL NO. _____ AND WAS AMENDED ON _____.

I HEREBY STATE THAT I HAVE REVIEWED AND UNDERSTAND THE CONTENTS OF THE ABOVE IDENTIFIED SPECIFICATION, INCLUDING THE CLAIMS, AS AMENDED BY ANY AMENDMENT REFERRED TO ABOVE. I ACKNOWLEDGE THE DUTY TO DISCLOSE INFORMATION WHICH IS MATERIAL TO PATENTABILITY AS DEFINED IN TITLE 37, CODE OF FEDERAL REGULATIONS §1.56.

I HEREBY CLAIM FOREIGN PRIORITY BENEFITS UNDER TITLE 35, UNITED STATES CODE §119, OF ANY FOREIGN APPLICATION(S) FOR PATENT OR INVENTOR'S CERTIFICATE LISTED BELOW AND HAVE ALSO IDENTIFIED BELOW ANY FOREIGN APPLICATION FOR PATENT OR INVENTOR'S CERTIFICATE HAVING A FILING DATE BEFORE THAT OF THE APPLICATION ON WHICH PRIORITY IS CLAIMED.

APPLICATION NUMBER	COUNTRY	DATE OF FILING	PRIORITY CLAIMED YES	NO

I HEREBY CLAIM THE BENEFIT UNDER TITLE 35, UNITED STATES CODE §120, OF ANY UNITED STATES APPLICATION(S) LISTED BELOW AND, INSOFAR AS THE SUBJECT MATTER OF EACH OF THE CLAIMS OF THIS APPLICATION IS NOT DISCLOSED IN THE PRIOR UNITED STATES APPLICATION IN THE MANNER PROVIDED BY THE FIRST PARAGRAPH OF TITLE 35, UNITED STATES CODE §112, I ACKNOWLEDGE THE DUTY TO DISCLOSE MATERIAL INFORMATION AS DEFINED IN TITLE 37, CODE OF FEDERAL REGULATIONS §1.56(A) WHICH OCCURRED BETWEEN THE FILING DATE OF THE PRIOR APPLICATION AND THE NATIONAL OR PCT INTERNATIONAL FILING DATE OF THIS APPLICATION.

APPLICATION NUMBER	DATE OF FILING	STATUS - PATENTED, PENDING OR ABANDONED

POWER OF ATTORNEY: AS A NAMED INVENTOR I HEREBY APPOINT AS MY ATTORNEY(S) WITH FULL POWER OF SUBSTITUTION AND REVOCATION, TO PROSECUTE THIS APPLICATION AND TRANACT ALL BUSINESS IN THE PATENT AND TRADEMARK OFFICE CONNECTED THEREWITH:

MARGARET A. HORN, REG. NO. 33,401
CHRISTOPHER L. STONE, REG. NO. 35,696
KIRSTEN A. ANDERSON, REG. NO. 38,813
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201

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POST OFFICE ADDRESS	POST OFFICE ADDRESS 1979 EATON AVENUE	CITY PALO ALTO	STATE OR COUNTRY CALIFORNIA	ZIP CODE 94070

202

FULL NAME OF INVENTOR	FULL FIRST NAME STUART	INITIAL	LAST NAME CAUSEY	
RESIDENCE & CITIZENSHIP	CITY PALO ALTO	STATE OR FOREIGN COUNTRY CALIFORNIA	COUNTRY OF CITIZENSHIP USA	
POST OFFICE ADDRESS	POST OFFICE ADDRESS 449 HAWTHORNE AVENUE #4	CITY PALO ALTO	STATE OR COUNTRY CALIFORNIA	ZIP CODE 94304

I FURTHER DECLARE THAT ALL STATEMENTS MADE HEREIN OF MY OWN KNOWLEDGE ARE TRUE AND THAT ALL STATEMENTS MADE ON INFORMATION AND BELIEF ARE BELIEVED TO BE TRUE; AND FURTHER THAT THESE STATEMENTS WERE MADE WITH THE KNOWLEDGE THAT WILLFUL FALSE STATEMENTS AND THE LIKE SO MADE ARE PUNISHABLE BY FINE OR IMPRISONMENT, OR BOTH, UNDER SECTION 1001 OF TITLE 18 OF THE UNITED STATES CODE, AND THAT SUCH WILLFUL FALSE STATEMENTS MAY JEOPARDIZE THE VALIDITY OF THE APPLICATION OR ANY PATENT ISSUING THEREON.

SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202
DATE	DATE

AGATCTACACAAGGAAATTGAAAAAATAGATAAAATTCGCAGGTATTAAGCCGACTAAAACAAATGAGTGAAGAA 80
 TCTAGATGTGTTCCGTTAACCTTTTATCTATTTAAAGCGTCATAATTCGGCTGAATTGGTTACTCACTTCTT

GAAAAGAAAAAAATAAACATATTTGAGTTAGTAAAAGAGAAAGAAAAATAAAAGAAGACCTCGGCTAACAGTCGA 160
 CTTTCTTTTTATTTATGTATAAAACTCAATCATTCTCTTTTATTTCTGGAGCCGAATTGTCAGCT

AAAACCAGAAATAATAAAAGAAAGAGACTGTGATTTTAATGGAAATCGTGAGGAAAAGAAAATTAAATTTTCAATT 240
 TTTGGTCTTATTATTTCTCTGACACTAAAATTACCTTAGCACTCCTTCTTTAAATTAAAGTAAA

CGAGGGATTAATTGTTGAAGTTGATGAAAATCTAGATAAAAATGCAGATCAAAATGTGTTGAATTGACATTATT 320
 GCTCCCTAATTAAACACATTCAACTACTTTAGATCTATTTTACGTCTAGTTTACACAACTTAAACTGTAATAA

GAAATACGTAGTATATCAATAATGGGGTTGTCTATTTATTTGCGAAGATTGAAAATCTGAGTGAAGAAAATAGTT 400
 CTTTATGCATCATAGTTATTACCCCCAACAGATAAAACGCTCTAACCTTAACTTACTCACTTCTTTATCAA

TGCGAGAGCAAAAAACCCCTGCCGTTTTCAATGACTTGGAAAAATTCTTGAGCGGTAGCGAAACTTTGAA 480
 ACGCTCTCGTTTTGGAACGGCAAAAAAGTTACTGAAACCTTTTAAAGTAACACTCGCCATCGCTTGAAACTT

ATTTTACATTGAAATTGAAAAATAAGGCAAAAGAACTCAAATGGAAAAATATTATAAAAAAGGAGATCG 560
 TAAAAAATGTAACCTTAAACTTTTATTCCGTTTCTTGAGTTACCTTTATAATAATATTTTCTCTAGC

Asn Phe Leu His Trp Phe Glu Lys Ile Arg Gln Lys Leu Lys Trp Lys Lys Tyr Tyr Lys Arg Arg Ser

GATATGGATTAAAAGCAGAAAATGACATTGAATGAAAAAGATTGGAAAAATCTATGCTGAGAGTGAATTAAA 640
 CTATACCTAAAATTTCGTCTTGACTGTAACTTACTTTCTAACCTTTAGATACGACTCTCACTTAATT

Asp Met Asp Phe Lys Ser Arg Lys Leu Thr Leu Asn Glu Lys Lys Asp Leu Glu Lys Ile Tyr Ala Glu Ser Glu Leu Lys

AGCAAAAAATTGGAACTCAACCCGGTGTGTTAGAAATGACGATGAAAGAAATGATGAAAAATATCAACCTCGATG 720
 TCGTTTTTAACCTTGAGTTGGGCCACAACAAATCTTACTGCTACTTTCTTACTACTTTATAGTTGGAGCTAC

Ala Lys Lys Leu Gly Thr Gln Pro Gly Val Val Leu Glu Met Thr Met Lys Asn Ile Asn Leu Asp

TTAATGAAGAACAGCAGGTCAATATAGGAAATTATTCAAAAATAAAGTTGAGCATAGTAAATCAGATGATCTAGTAACG 800
 AATTACTTCTTGTCGTCAGTTATCCTTAAAGTTTATTCGACTCGTATCATTAGTCTACTAGATCATTG

Val Asn Glu Glu Thr Ala Gly Gln Tyr Arg Lys Leu Phe Lys Asn Lys Val Glu His Ser Lys Ser Asp Asp Leu Val Thr

FIGURE 1A

GGACTATTAGAGTGTGAACTCGAAATAGTTGATAAAAACAAGAAGTGCCTTCGTTTGATTTGTGAGAGAATTCA 880
 CCTGATAATCTCACACCTTGAGCTTATCAAAACTATTTGTTCTCACGGAAAGCAAAACATAAACACTCTCTTAAGT
 Gly Leu Leu Glu Cys Gly Thr Arg Asn Ser Phe Asp Lys Thr Arg Ser Ala Phe Arg Phe Cys Ile Cys Glu Arg Ile Gln
 GCAACTGAGAAAAGAAGCTGATAATGCAAGAAGAGTAAAGATTCGATACAATGAAAGCAAAACTAAAGAGGCTTTG 960
 CGTTGACTCTTCTTCGACTATTACGTTCTCTCATTTCTAAAGCTATGTTACTTCGTTTGATTTCTCCGAAAC
 Gln Leu Arg Lys Glu Ala Asp Asn Ala Arg Arg Val Lys Asp Phe Asp Thr Met Lys Ala Lys Thr Lys Glu Ala Phe
 AATTGAGTTTGTTTGATAAGGATTTTGAGTGAAAATAGAATTCAATGGAATGATATTCACAACAAAAAGAC 1040
 TTAACTCAAAACAAAACATTCTAAAGACTCACTTTATCTTAAGTTACCTACTATAAAGAGTGTGTTTTCTG
 Glu Leu Ser Phe Val Phe Asp Lys Asp Phe Leu Ser Glu Asn Arg Ile Gln Trp Asn Asp Ile Ser His Asn Lys Lys Asp
 TCTGCAAGTAAAAGAAAAACATGAAAGAAGCGGACACAATGGATGATATTTAAAGAGGCTAAAAATAATAATCTAC 1120
 AGACGTTCTTTCTTTGTTACTTCGCGCTGTGTTACCTACTATAAAATTCTCCGATTTTATTATTTAGATG
 Ser Ala Ser Lys Arg Lys Thr Met Lys Glu Ala Asp Thr Met Asp Asp Ile Phe Lys Arg Leu Lys Asn Asn Lys Ser Thr
 ATATGATCGTTATGCTGGATTCCCTTCTATTGTTGATTACAGGTTGCAGACCAGCAGAAGTTAAAGGGTATAGAGA 1200
 TATACTAGCAATACGACCTAAGGAAAGATAAACAGCTAATGTCCAACGTCGGTCGTTCAAATTCCCATATCTCT
 Tyr Asp Arg Tyr Ala Gly Phe Leu Ser Ile Cys Ser Ile Thr Gly Cys Arg Pro Ala Glu Val Leu Lys Gly Ile Glu
 TAGTAAGAACAGATATGAGGATGGTATATCTTTAAAATACTTGGTCAAAGGTTGGAAATGACAGAGGGCAAAGCGAA 1280
 ATCATTCTTGCTATACTCCTACCATATAGAAAATTGAAACCACGTTCAACCTTACTGTCTCCGTTCGCTT
 Ile Val Arg Asn Arg Tyr Glu Asp Gly Ile Ser Phe Lys Ile Leu Gly Ala Lys Val Gly Asn Asp Arg Gly Gln Ser Glu
 AGAACATTACATTTGATTTATCAAAATATCATGATAATGAGCAAATGAATTATTTGTCGAATTAAAAGATAATAA 1360
 TCTTGTAATGTAACAAATAGTTTATAGTACTATTACTCGTTACTTAATATAAACAGCGTTAATTCTATTATT
 Arg Thr Leu His Phe Asp Leu Ser Lys Tyr His Asp Asn Glu Gln Met Asn Tyr Ile Leu Ser Gln Leu Lys Asp Asn Lys
 ATTTTCTACAAACCAGATGGAAAGCTCTACAAACAGCTTGAGGCAATACCTCTACATCCAACATAGAACGTTTCACTGT 1440
 TAAAAAGATGTTGGTCTACCCCTCGAGATGTTGCGACTCCGTTATGGAGATGTAGGTTATCTGCAAAAGTGACA
 Phe Phe Tyr Lys Pro Asp Gly Lys Leu Tyr Asn Ser Leu Arg Gln Tyr Leu Tyr Ile Gln His Arg Thr Phe Ser Leu
 ATACACTTCGTACAGGGTTGCAGGTGATCTCAAGGCATCCGGTGCAGATGACTTCACCATAGCGGCTNTTTGGGTCA 1520
 TATGTGAAGCAGTGTCCCAACGCTCACTAGAGTTCCGTAGGCCACGTCTACTGAAGTGGTATCGCCGANAAAACCCAGTG
 Tyr Thr Leu Arg His Arg Val Ala Ser Asp Leu Lys Ala Ser Gly Ala Asp Asp Phe Thr Ile Ala Ala ??? Leu Gly His

FIGURE 1B

AGAGTGACTCAAAGCCAGGAGTTACTACGGCTATGCTCGTCGNAAGGTGGTATCGCTGTA
TCTCACTGAGTTCGGTCCCTCAATGATGCCGATACGAGCAAGCAGCNTCCACCAAGCGACATTGACCACAACTCACGA 1600
Arg Val Thr Gln Ser Gln Glu Leu Leu Arg Leu Cys Ser Phe Val ?? Arg Trp Tyr Arg Cys Asn Trp Cys
CTGATGTTGTGAAAGCAAACAAGAGTCAGTTNGCTGTATCAAGGACTCCGAGCCAGATCT 1660
GACTACAACACTTCGTTGTTCTCAGTCAANCACATAGTTCCCTGAGGCTCGGTCTAGA

FIGURE 1C

AGATCTAACCAAGTTAAAATCGCACTCAAGAAGTAAAAAATAGGGGCCGGCACCGGCTCTTTTTGGTGTGGTAG 80
 TCTAGAGTTGGTCAAATTTAGCGTGAAGTCTCATTATCCCCGGCCGTGGCGAGAAAAAAACCAACAAACATC

TTAGTGGATATCTGTTAGCTACAGAGAAAAGCGATTTAGAGGGTTGACGAGGTTTCAGCTATCCAGGGTTT 160
 AATCACCTATATAGACAATCGATGTCCTTCGCTAAATCTCCAAACTGCTCCAAAAGCTCGATAGGTCCAAA

TGGGTTTTGGGTTGGATCAGAAAAGTCGTTCAAGATTATTGACATAAAGACAGGAAGGTTATAACAAGTACCA 240
 ACCCAAAAACCCAACCTAGTCTTCAGCAAGTTCTAATAACTGTATTCTGTCTTCCAAATATTGTTATGGTCTAT

CGACAAAACCAGCTTGCAGGCTGGCTTGAAGGACTAAAGAAGTGGGGACTTCTTGAGTCTTGTAAATCAAGTTGGTC 320
 GCTGTTTGGTCGAAACGTCCGACCGAAACTCCTGATTTCTCACCCCTGAAGAAACTCAGAACATTAGTTCAACCA

AGAACTCGATTACGATTGTAAAGTAGAAATCTAACTCACATTGCGAGAAAGTCAAACCTACCTCTTAGTTACAAC 400
 TCTTGAGCTAATGCTAACATTCTAGTTAGATTGAGTGTAAAGCGTCTTCAGTTGAATGGAGAATCAATGTTGAGTT

AAATTCCTAGCCTTTAGATCCTAACATACATATTTGTTAAACCGATTGTGTCCGGTGGGGAGCCAT 480
 TTAAAGGATCGGAAAAGTCTAGGAATTCGTATGTATAAAACAAATTGGCTAACACAGGCCACAAACACACCTCGGT

TGATCCGAGTGGTCAATATGTGATTGTTGCCAACAGTGTATGTAGGTCTAACCGGGAGTGCTACAAAGACCATACC 560
 ACTAGGCTCACCAAGTTACACTAACAGCGTTGTCACATACATCCAGATTGCCCTCACGATTTCTGGTATGG

CGAAACGAGTGCCTAAGTGTGGTTATCAACCAGGTAAGCTATGAGAAAGCCCAGCCATAATGGGGTTAGGTTGAAG 640
 GCTTGCTACGGATTACAAACCAATAGTTGGTCATTGATACTCTTCGGTCGGTATTACCCAACTACAGGAGATTGAA

CAAGTCTTCATATGGTGCACACAAGGGGTGTAAGTAGGGTGTGTCGTCACCTGATAACTTTCACTTTGAGTGAAATT 720
 GTTCAGAAGTATACCACGCTGTGTTCCCCACATCATCCCACAGCAGTTGACTTCCAAACTATCGAGATTGAAACAC

TCTGTGGGTCAAGCCTCAAGTGTGATCTGTGGTGTGTCGTCACCTGATAACTTTCACTTTGAGTGAAATTCAAGGAGG 800
 AGACACCCAGTTGGAGTTACGACTAGACACACAGCAGATGGACTATTGAAAGTGAAAAGCTCACTTTAAGTCCTCC

CGAAACTATGGGTCAAGCCCAGCTTGCTGGGTTCGGCACATCCAGCTACAGCATTGGTGTCTTGCAGCTGAAGC 880
 GCTTGATACCCAGTTGGGTGAAACGACCCAAAGCCGTGAGGTGAATGTCGTAACCACGAGAACGCTTCGACTTCG

FIGURE 1D

AAAAAAATCTAATCCAGGGTTGGGTTTTATACCAGAACAAACAAAAAAATAAAACAAAGAAAAATTTCGAGCGA 960
 TGTTTTAGATTAGGTCCAAACCCAAAAATGGTCTCGTTGTTTTATTTGTTTTAAAAGCTCGCT

AAAAATATTTGGAATTTAAAGGCATACTGCTACCGCACTTGCATATTAAAACCTGACTATCTTATAAGT 1040
 TTTTATAAAACCTAAAAAATTCGCTATGAACGATGGCGTAAACGGTATAAATTGGACTGATAGAAATATTCA

TAATAGATATATCCGTTAGATTATAAGTATGTTAAAACGAGTAAAACAATAACTTATATATTAAATTCTGAATTATA 1120
 ATTATCTATATAGGCAATCTAATATTCATACAATTGCTATTGTTATTGAATATATAAAATTAAAGACTTAATAT

TTTGACAGTGATTATTAATATTAAGAGATATCTATTAGCTAAATATAACTAAAAAGAGGTAAATATGGAT 1200
 AACTGTCACTAATAAATTATAATTCTCTATAGATAATCGAATTATATTGATTTCTCCATTATACCTA

TGTGATTTAAAAAGCATTAGAAAATGAAATAGAACATTATAAAAAGACGGTATCAAATCTTACAATACCT 1280
 ACACATAAATTTTCGTAATCTTACTTCTGTAATATTTCTGCCACTATAGTTAGAAAGAATGTTATGAA

GCATTACTTGATATAGATAAAGCATTAAATGGTATGAATGTGGCGATATTATAAACTCAAATTATCCATTGATGAA 1360
 CGTAATGAAACTATATCTATTCGTAATTACCACTACTTACACCGCTATAATTGAGTTAAAGGTAACTACTT

GTTTGATCTCTTGATGTTGAGCACAATTTCGGCTGGCTTCAATAAAATAACAGAGACGAAATGAATATTATCA 1440
 CAAAACTAGAAGAACTACAACCTCGTAAAGCCGACCCGAAAGTTATTATGTCTGTTACTTATAAAATAGT

TCAGCTAAAATGATTTAAAAACTCGTTTTATTCAATTGATCAATTGGAAAGAATTAAATTACGATGA 1520
 AGTCGATTTGACTTTACTAAATTTTATGAGCAAGAAATAAGTAAGCTAGTTAACCTCTTAAATTGCTACT

GATGAGTACAATACATCAAGAAATGATTAAGGATTAGATAATTACACATATGGAGAAATAACCATATGAATAATAAAAT 1600
 CTACTCATGTTATGTAGTTCTTACTAATTCTTAATCTATTGTATACCTCTTATTGGTATACTTATTATTTA

AAGAGAATATATTGATTCGAAATAACAAAGATATAAAAGAAAGTCAGCTTAAATATCTGCATTGATCGATGTT 1680
 TTCTCTTATATAACTAAAGCTTATTGTTCTATATTCTTACGGATACGAAAGGCGTAATTGTTGTTCTT

TAAAAGTAGATGAAAATTATTGATGAAGAGGGATTGCAACTAAAGATATTGAAAATATCGTATGAAAATCCTATTGAT 1760
 ATTTCATCTACTTTAAATAACTACTTCTCTAAACGTTGATTCTATAACTTTATAGCATACTTTAGGATAACTA

GATCCAGATGATGGCATAAGAAAATCACAATTGCAACGAAGAAATGCCTATGCTTCCGCATTAAAAACAAAGCAAAA 1840
 CTAGGTCTACTACCGTATTCTTAGTGTAAACGCGTCTTACGGATACGAAAGGCGTAATTGTTGTTCTT

FIGURE 1E

GAGATCT
→ 1847
CTCTAGA

FIGURE 1F

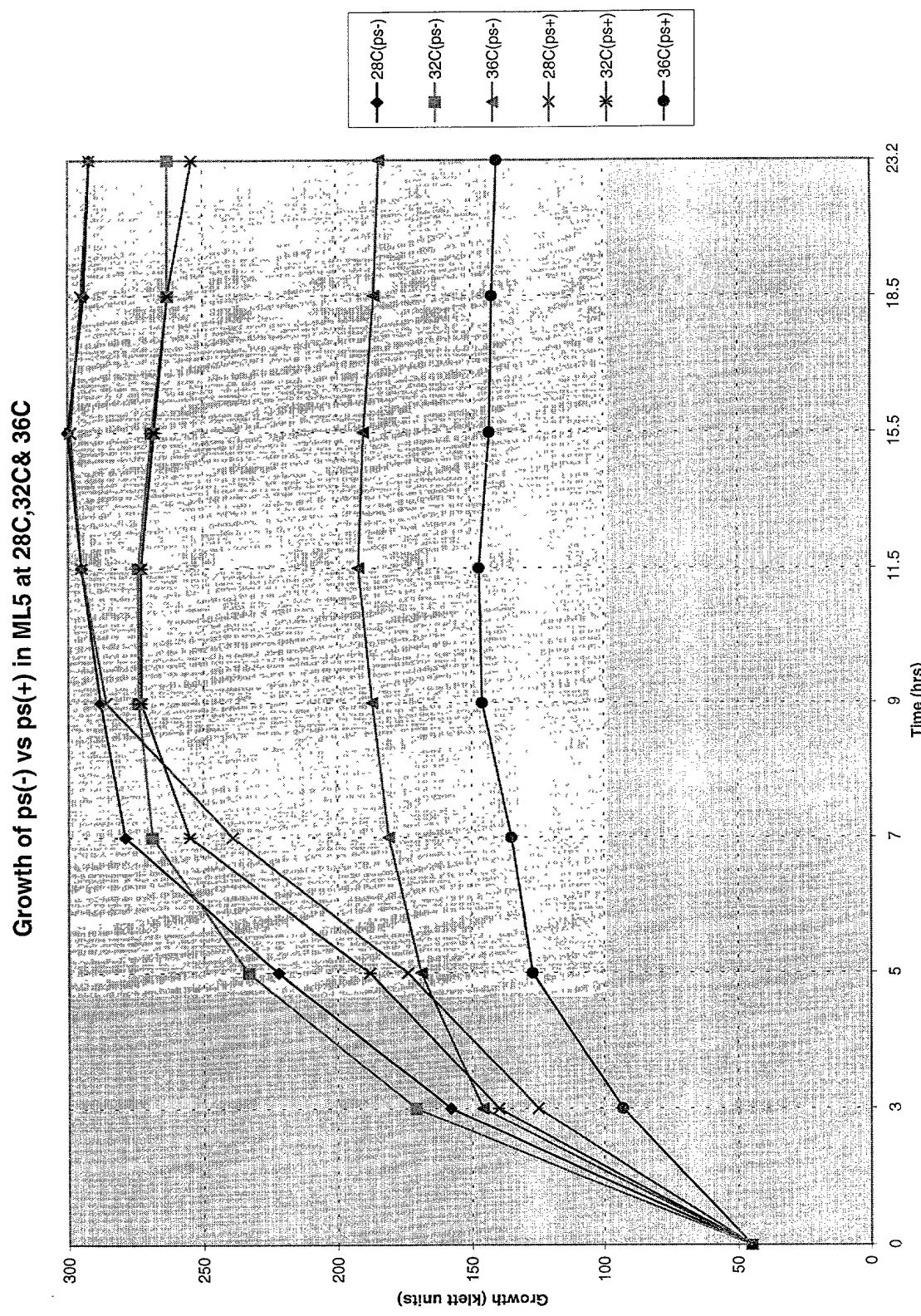


FIGURE 2

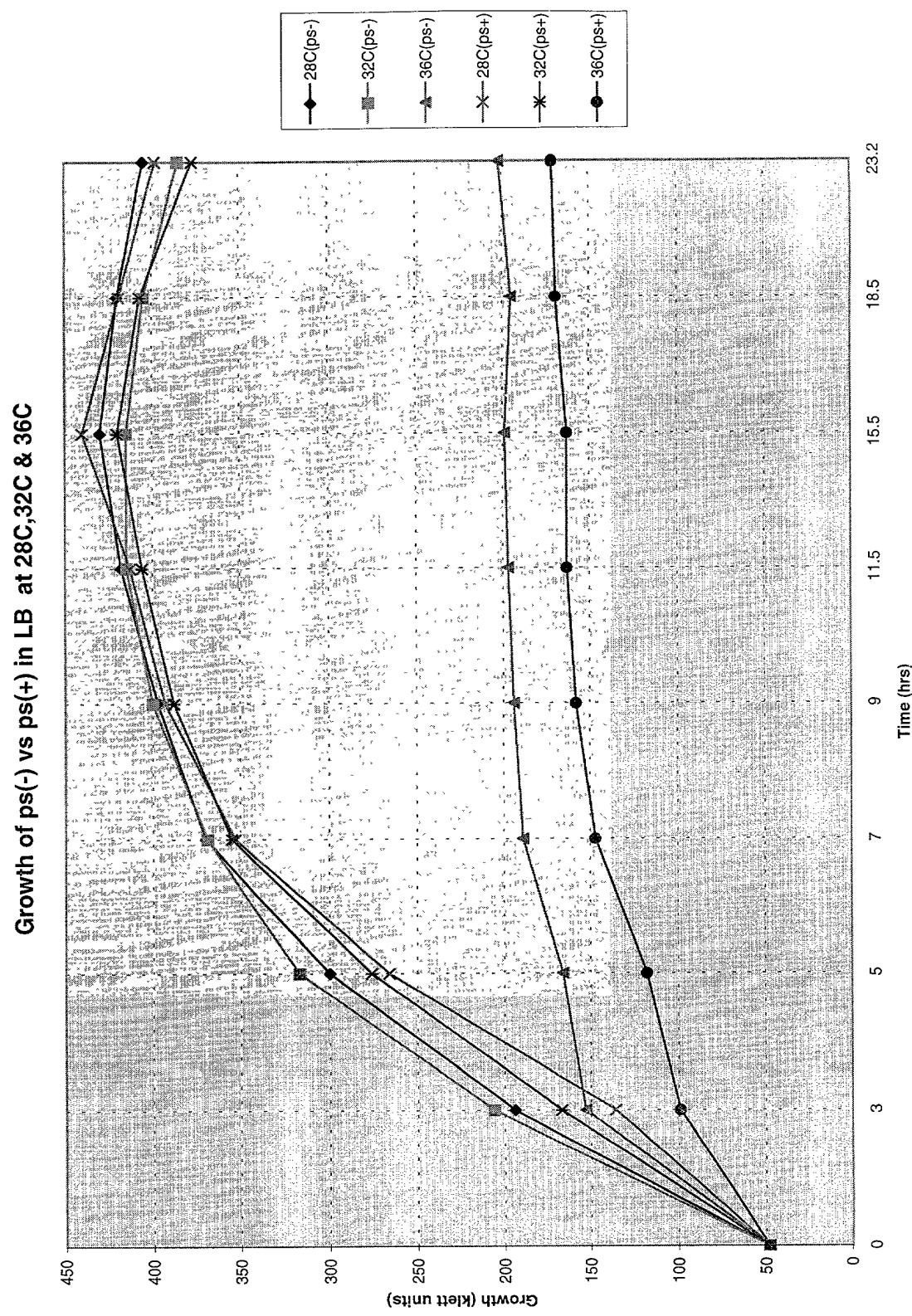


FIGURE 3